

RESEARCH PAPER

Exogenous administration of thiosulfate, a donor of hydrogen sulfide, attenuates angiotensin II-induced hypertensive heart disease in rats

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BACKGROUND AND PURPOSE

Hypertension is an important mediator of cardiac damage and remodelling. Hydrogen sulfide (H₂S) is an endogenously produced gasotransmitter with cardioprotective properties. However, it is not yet in clinical use. We, therefore, investigated the protective effects of sodium thiosulfate (STS), a clinically applicable H₂S donor substance, in angiotensin II (Ang II)-induced hypertensive cardiac disease in rats.

EXPERIMENTAL APPROACH

Male Sprague Dawley rats were infused with Ang II (435 ng kg min⁻¹) or saline (control) for 3 weeks via s.c. placed osmotic minipumps. During these 3 weeks, rats received i.p. injections of either STS, NaHS or vehicle (0.9% NaCl).

KEY RESULTS

Compared with controls, Ang II infusion caused an increase in systolic and diastolic BP with associated cardiac damage as evidenced by cardiac hypertrophy, an increase in atrial natriuretic peptide (ANP) mRNA, cardiac fibrosis and increased oxidative stress. Treatment with NaHS and STS prevented the development of hypertension and the increase in ANP mRNA levels. Furthermore, the degree of cardiac hypertrophy, the extent of histological fibrosis in combination with the expression of profibrotic genes and the levels of oxidative stress were all significantly decreased.

CONCLUSIONS AND IMPLICATIONS

Ang II-induced hypertensive cardiac disease can be attenuated by treatment with STS and NaHS. Although BP regulation is the most plausible mechanism of cardiac protection, the antifibrotic and antioxidant properties of released sulfide may also contribute to their effects. Our data show that H₂S might be a valuable addition to the already existing antihypertensive and cardioprotective therapies.

LINKED ARTICLES

This article is part of a themed section on Pharmacology of the Gasotransmitters. To view the other articles in this section visit <http://dx.doi.org/10.1111/bph.2015.172.issue-6>

Abbreviations

Ang II, angiotensin II; ANP, atrial natriuretic peptide; CBS, cystathionine β -synthase; CSE, cystathionine γ -lyase; Nox2, NADPH oxidase 2 (also known as Cybb, cytochrome b (558) subunit β); DBP, diastolic BP; H₂S, hydrogen sulfide; K_{ATP} channels, ATP-sensitive potassium channels; MDA, malondialdehyde; NaHS, sodium hydrosulfide; NOx, nitrite and nitrate; RAAS, renin-angiotensin-aldosterone system; ROS, reactive oxygen species; (S)TS, (sodium) thiosulfate (Na₂S₂O₃); SBP, systolic BP; WGA, wheat germ agglutinin

Table of Links

TARGETS	LIGANDS
H ₂ S	Angiotensin II
K _{ATP} channel	Atrial natriuretic peptide
AT ₁ receptor	Galectin-3
	Fibronectin
	Nitric oxide
	NaHS

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a, b, c).

Introduction

Hypertensive heart disease is a major global health problem and a considerable cause of cardiovascular morbidity and mortality. Hypertension is a key contributory factor in cardiovascular disease, especially cerebrovascular disease and heart failure (Drazner, 2011). The heart's response to increased stress such as hypertension is captured by the term 'cardiac remodelling', which encompasses myocyte hypertrophy, formation of cardiac fibrosis and vascular rarefaction. Remodelling is associated with activation of various stress responses including the induction of neurohormonal and inflammatory pathways. Progressive remodelling may perpetuate into left ventricular dysfunction and heart failure (Mann, 2002; Jia *et al.*, 2012). Angiotensin II (Ang II) is a well-established mediator in the pathogenesis of hypertensive heart disease. In addition to its hypertensive effect, Ang II induces cardiac remodelling by directly exerting prohypertrophic effects through enhancing inflammatory responses and activation of several profibrotic factors via a number of signalling pathways (Wynn, 2008; Shahbaz *et al.*, 2010). Blockade of the renin-angiotensin-aldosterone system (RAAS) is able to regress cardiac remodelling and has evolved into the cornerstone of cardiovascular disease management. Despite the use of RAAS intervention and other antihypertensive treatments, many patients with hypertensive heart disease still develop heart failure (Koitabashi and Kass, 2011). Therefore, additional modes of intervention are warranted.

Hydrogen sulfide (H₂S) is recognized as a biologically important gaseous signalling molecule with a myriad of physiological functions (Wang, 2002). H₂S is endogenously produced from the degradation of L-cysteine by cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) (Abe

and Kimura, 1996; Singh *et al.*, 2009) and from D-cysteine by 3-mercaptopyruvate sulfurtransferase (Shibuya *et al.*, 2013). H₂S is serially oxidized to persulfide, thiosulfate (TS, S₂O₃), sulfite and sulfate (Hildebrandt and Grieshaber, 2008; Kabil and Banerjee, 2010). Similar to NO, H₂S functions as an endothelial cell-derived relaxing factor via direct activation of ATP-sensitive potassium (K_{ATP}) channels (Mustafa *et al.*, 2011). Accordingly, deprivation of endogenously produced H₂S contributes to the development of hypertension (Yang *et al.*, 2008; Sen *et al.*, 2010). Furthermore, H₂S has beneficial effects on oxidative stress (Bos *et al.*, 2013), inflammation and fibrosis (Snijder *et al.*, 2013). In various cardiovascular disease states, a reduction in H₂S plasma and tissue levels was observed, suggesting a possible role for H₂S in its pathogenesis (Jiang *et al.*, 2005; Zhao *et al.*, 2008; Kovacic *et al.*, 2012; Kondo *et al.*, 2013). Exogenous treatment with H₂S is cardioprotective in various experimental models of cardiac injury. The pathways implicated in the cardioprotective actions of H₂S are multiple. While most of the studies focus on acute myocardial protection, there are some elegant studies investigating the protective effects of H₂S in experimental models for chronic heart failure (Szabo *et al.*, 2011). The effects of H₂S on Ang II-induced hypertensive heart disease have not been investigated.

For the administration of H₂S in experimental models, soluble sulfide salts, such as NaHS and Na₂S, are often used. Other ways of delivery include H₂S gas or slow-release H₂S donors. A novel approach is the use of sodium thiosulfate (STS, Na₂S₂O₃), an endogenous player in the enzymatic pathways of H₂S production in mammalian cells. Increasing evidence supports the idea that a dynamic conversion exists between STS and H₂S (Ubuka *et al.*, 2001; Olson *et al.*, 2013). In humans, the short-term therapeutic use of STS has been

proven safe for the treatment of calciphylaxis (Singh *et al.*, 2011), providing us with the possibility of using H₂S related therapies in a clinical setting.

The vasodilating and cytoprotective features of H₂S make it an attractive candidate for therapeutic reduction of the cardiovascular alterations associated with hypertension. We investigated the protective properties of sulfide-releasing compounds in Ang II-induced hypertensive heart disease in rats.

Methods

Animals

Male Sprague Dawley rats ($n = 27$, 240–280 g, Harlan, Zeist, the Netherlands) were housed under standard conditions with a 12 h light-dark cycle at the animal research facility with *ad libitum* access to food and water. Experimental procedures were in agreement with institutional and legislator regulations and approved by the local committee for animal experiments (DEC 6412B). With regard to the execution of our animal experiments, we followed the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Ang II infusion and NaHS or STS treatment

Osmotic minipumps (model 2004, Alzet, Cupertino, CA, USA) were placed s.c. under general anaesthesia (2% isoflurane/O₂) for continuous administration of Ang II (435 ng kg⁻¹ min⁻¹, $n = 7$ per group; Bachem, Weil am Rhein, Germany) or vehicle (0.9% NaCl, $n = 6$). To check the depth of the anaesthesia a pain stimulus was administered. Postoperatively, all rats received a s.c. injection of 50 µg kg⁻¹ buprenorphin (Schering-Plough, Houten, the Netherlands) for analgesic purposes and were allowed to recover from the surgery at 37°C in a ventilated incubator. During placement of the pumps, Ang II-infused rats were randomized to either STS (1 g kg⁻¹ day⁻¹; Sigma, Zwijndrecht, the Netherlands), NaHS (5.6 mg kg⁻¹ day⁻¹; Sigma) or 0.9% NaCl treatment. Rats receiving 0.9% NaCl via osmotic minipumps received treatment with 0.9% NaCl. During the 3 weeks of infusion, rats received i.p. injections with one of the compounds twice a day. At baseline, blood was collected via orbital puncture. On a weekly basis, body weight was measured. After 3 weeks, BP was measured under general anaesthesia (2% isoflurane/O₂) via an intra-aortic probe (Cardiopap/5, GE Healthcare, Little Chalfont, Buckinghamshire, UK). Subsequently, rats were terminated and blood was collected. Hearts were perfused with 0.9% NaCl, removed and then weighed. The hearts were dissected and mid-papillary slices were fixed in 4% paraformaldehyde and paraffin embedded for immunohistochemical analysis, upper parts were immediately snap frozen in liquid nitrogen and stored at -80°C for molecular analysis.

Cardiomyocyte size

To investigate cardiac hypertrophy, we visualized the membrane of the cardiomyocytes and measured its size. After deparaffinization, mid-papillary cardiac sections were incubated with wheat germ agglutinin (WGA)-FITC (1:100, L4895-2 mg, Sigma) for 30 min in room temperature. Nuclei were stained with 4,6-diamino-2-phenylindole (6-H-1200, Vector Laboratories, Brunswick Chemie, Amsterdam, The

Netherlands). Images were captured with a fluorescence microscope. For each slide, the size (short axis) of 75–100 WGA-stained cardiomyocytes was measured with NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cardiac fibrosis

To investigate cardiac fibrosis Masson's trichrome staining was used on paraffin-embedded mid-papillary cardiac sections. Subsequently, cardiac sections were scanned using an Aperio Scanscope GS (Aperio Technologies, Vista, CA, USA). The extent of collagen deposition was determined using the Aperio positive pixel analysis v9.1 algorithm. The ratio between the fibrotic surface area and the total cardiac surface area was calculated. Histopathological analysis was performed in a blinded fashion.

Qualitative real-time PCR (qRT-PCR)

Rat cardiac tissue was homogenized in lysis buffer and total RNA was extracted using the TRIZOL method (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using Superscript II with random hexamer primers (Invitrogen). Gene expression (Applied Biosystems, Foster City, CA, USA) was determined by qRT-PCR based on the Taqman methodology. GAPDH was used as a housekeeping gene. The primers of atrial natriuretic peptide (ANP) and Cybb (Nox2) were obtained from Applied Biosystems as Assays-on-Demand (AOD) gene expression products. The AOD IDs used were: Cybb (Nox2) Rn00576710_m1 and Nppa (ANP) Rn00561661_m1. The primers used for fibronectin and galectin-3 were: FN1 (fibronectin) Forward – GTACCACTGGCCACACCTAC Reverse – TGTCAGCCTGCACGTCCAAC; Lgals3 (galectin-3) Forward – CCCGCTTCAATGAGAACAAC Reverse – ACCG CAACCTTGAAGTGGTC (both from Sigma). For Cybb (Nox2) and ANP, the qRT-PCR reaction mixture contained 20 ng cDNA template and 5 µL PCR mastermix. Nuclease-free water was added to a total volume of 10 µL. For fibronectin and galectin-3, qRT-PCR was performed in a volume of 20 µL containing 10 ng cDNA and 15 µL PCR mastermix (SYBR GREEN Applied Biosystems; 5 mL P/N 4309155). All assays were performed in triplicate. The thermal profile was 2 min at 50°C, 15 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The average Ct values for target genes were subtracted from the average housekeeping gene Ct values to yield the δ Ct. Results were expressed as 2^{- δ Ct}.

Plasma nitrite and nitrate (NOx)

Measurement of combined NOx was used as a marker for NO production. We colorimetrically measured plasma NOx applying the Griess reaction after reduction of nitrate to nitrite.

Urinary malondialdehyde (MDA) measurements

MDA is a major breakdown product of lipid peroxides and generated after oxidative stress. Twenty microlitres of urine was incubated with 90 µL of 3% SDS and 10 µL of 0.5 M butylated hydroxytoluene followed by the addition of 400 µL 0.1 N HCl, 50 µL 10% phosphotungstic acid and 200 µL 0.7% 2-thiobarbituric acid. The reaction mixture was incubated for 30 min at 95°C. After adding 800 µL of 1-butanol, the

samples were centrifuged at 960 g for 10 min. Two hundred microlitres of the 1-butanol phase was fluorescently measured using 530 nm excitation and 590 nm emission wavelengths.

Plasma and urinary calcium measurements

Plasma and urinary calcium levels were determined by standard assays (Roche Diagnostics GmbH, Mannheim, Germany) in our clinical chemical laboratory.

Statistical analysis

Data were analysed and graphed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). All data are expressed as the mean \pm SEM unless otherwise indicated. Normality was tested using the Kolmogorov–Smirnov test. Statistical analyses were performed using *t*-tests, Mann–Whitney *U*-tests, two-way ANOVA, one-way ANOVA or Kruskal–Wallis tests where appropriate. Bonferroni or Dunn's post-correction was applied where multiple comparisons were made. Statistical significance was accepted at $P < 0.05$.

Results

STS and NaHS treatment attenuates Ang II-induced hypertension

Infusion with Ang II significantly increased systolic BP (SBP; 211 ± 9 mmHg vs. 143 ± 2 mmHg, $P < 0.001$) and diastolic BP (DBP; 127 ± 10 mmHg vs. 84 ± 2 mmHg, $P < 0.01$) compared with controls. Simultaneous treatment with either STS or NaHS decreased SBP by 18% (173 ± 7 mmHg, $P < 0.001$) and 22% (164 ± 3 mmHg, $P < 0.001$), and DBP by 30% (89 ± 7 mmHg, $P < 0.01$) and 26% (93 ± 8 mmHg, $P < 0.05$) respectively (Figure 1, Table 1).

Effects of STS and NaHS on cardiac hypertrophy

After 3 weeks, Ang II-infused rats treated with vehicle had a lower body weight compared with NaCl-infused controls ($P < 0.001$). Treatment with NaHS, but not with STS, resulted in a higher bodyweight compared with treatment with vehicle (NaHS: $P < 0.01$; Figure 2A). The heart-to-body weight ratio of vehicle-treated Ang II-infused rats was significantly increased compared with 0.9% NaCl-infused controls ($P < 0.001$) (Figure 2B), suggesting that these animals developed hypertrophy subsequent to Ang II infusion. To confirm this, we investigated the cardiomyocyte size by analysing WGA-stained cardiac sections. Indeed, we observed cardiomyocyte hypertrophy in the vehicle-treated Ang II-infused rats as evidenced by an increased cardiomyocyte size ($P < 0.001$) (Figure 2C and D). Treatment with STS and NaHS prevented the development of cardiac hypertrophy as evidenced by a preserved heart-to-body weight ratio (STS: $P < 0.05$, NaHS: $P < 0.01$) and cardiomyocyte size ($P < 0.001$) (Figure 2, Table 1).

Treatment with STS and NaHS reduces ANP mRNA levels

ANP mRNA expression, a hallmark of the reactivation of the fetal gene programme, was significantly increased in hearts of

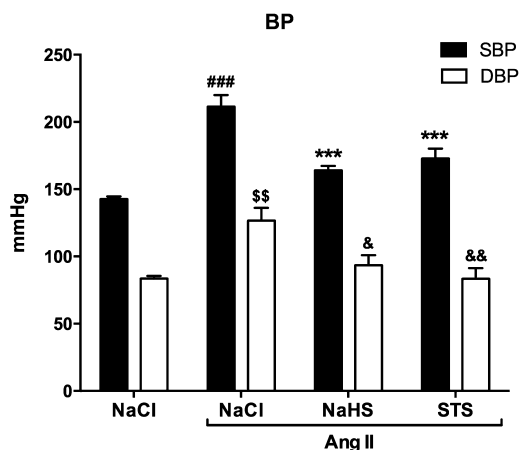


Figure 1

Treatment with STS and NaHS prevents the development of Ang II-induced hypertension. Three weeks of Ang II infusion caused hypertension in the vehicle treated animals ($n = 7$). SBP was increased by 48% and DBP by 51%. Treatment with STS ($n = 7$) and NaHS ($n = 6$) prevented the development of hypertension by Ang II. (SBP: ### $P < 0.001$ vs. control, *** $P < 0.001$ vs. Ang II + NaCl; DBP: \$\$\$ $P < 0.01$ vs. control, && $P < 0.01$ vs. Ang II + NaCl, & $P < 0.05$ vs. Ang II + NaCl).

Ang II-infused rats treated with vehicle compared with 0.9% NaCl-infused controls ($P < 0.01$). In hearts of STS- and NaHS-treated rats, the relative ANP expression was significantly reduced compared with vehicle-treated rats ($P < 0.05$) (Figure 3, Table 1).

Rise in plasma NOx after infusion with Ang II

After 3 weeks of infusion with Ang II, plasma NOx levels were increased by 71% compared with 0.9% NaCl-infused control animals (23.2 ± 1.7 $\mu\text{mol L}^{-1}$ vs. 13.6 ± 0.3 $\mu\text{mol L}^{-1}$, $P < 0.001$). In the STS- and NaHS-treated groups, plasma NOx levels were lower compared with NOx levels in Ang II-infused rats treated with vehicle (STS: 19.0 ± 0.9 $\mu\text{mol L}^{-1}$, NaHS: 16.3 ± 1.2 $\mu\text{mol L}^{-1}$) (Figure 4, Table 1).

Protective effects of STS and NaHS against cardiac fibrosis

Fibrosis, as measured by collagen deposition in Masson stained sections 3 weeks after Ang II infusion, was markedly increased in animals treated with vehicle compared with 0.9% NaCl-infused controls ($P < 0.05$). Treatment with STS or NaHS reduced collagen deposition by 52% for both compounds (STS: $P < 0.01$, NaHS: $P < 0.05$) (Figure 5, Table 1). Cardiac mRNA levels of the profibrotic genes fibronectin and galectin-3 were assayed. Fibronectin was massively increased in Ang II-infused animals treated with vehicle ($P < 0.001$), while only a moderate increase was detected in animals of the STS- and NaHS-treated groups (Figure 6A, Table 1). Galectin-3 mRNA expression was increased 3.2-fold in hearts of vehicle-treated Ang II-infused rats compared with control ($P < 0.001$). In the hearts of STS- and NaHS-treated mice, the relative

Table 1

Overview of all parameters

	NaCl (%)	Ang II + NaCl (%)	Ang II + NaHS (%)	Ang II + STS (%)
SBP	100 (1)	148 (6)###	115 (2)***	121 (5)***
DBP	100 (2)	146 (11)##	108 (9)*	103 (10)**
Body weight week 3	100 (7)	70 (2)###	88 (5)*	85 (5)
Heart-to-body weight ratio	100 (2)	160 (3)###	125 (9)**	134 (7)*
Cardiomyocyte size	100 (1)	139 (4)###	104 (2)***	101 (3)***
ANP mRNA	100 (10)	928 (255)##	332 (103)*	274 (63)*
NOx plasma	100 (2)	171 (13)###	119 (25)**	140 (6)*
Fibrosis	100 (20)	222 (43)#	108 (11)*	107 (9)**
Fibronectin mRNA	100 (12)	429 (33)###	229 (57)**	230 (59)*
Galectin-3 mRNA	100 (16)	320 (27)###	178 (29)**	228 (22)*
Nox2 mRNA	100 (7)	201 (26)##	121 (17)**	140 (11)*
MDA excretion	100 (14)	237 (18)###	154 (25)*	167 (10)*
Plasma calcium	100 (4)	104 (6)	103 (5)	109 (3)
Calcium excretion	100 (44)	567 (44)#	342 (110)	698 (150)

The results are presented as % change relative to control (NaCl). ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$ vs. NaCl; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs. Ang II + NaCl. SEM is shown in parentheses.

galectin-3 expression was reduced by 29% ($P < 0.05$) and 44% ($P < 0.01$) respectively (Figure 6B, Table 1).

Treatment with STS and NaHS reduces oxidative stress

Cybb (Nox2) is a member of the NADPH oxidase family that is responsible for the formation of reactive oxygen species (ROS). Cybb (Nox2) mRNA expression is increased twofold after Ang II infusion ($P < 0.01$) (Figure 6A). Also, the urinary excretion of MDA, which is considered a biomarker of oxidative stress, is increased more than twofold in Ang II rats treated with vehicle ($P < 0.001$) (Figure 6B). Simultaneous treatment with either STS or NaHS resulted in a decrease in Cybb (Nox2) mRNA expression by 30% ($P < 0.05$) and 40% ($P < 0.01$), and urinary MDA levels by 30% ($P < 0.05$) and 35% ($P < 0.05$) respectively (Figure 7, Table 1).

Unchanged plasma and urinary calcium levels

No significant differences between groups were observed in plasma calcium levels. Urinary excretion of calcium was increased in Ang II-infused rats treated with vehicle compared with 0.9% NaCl-infused controls ($P < 0.05$). In both STS- and NaHS-treated groups, urinary calcium levels were not different from vehicle-treated Ang II-infused animals (Figure 8, Table 1).

Discussion

In the present study, we demonstrated that exogenous administration of STS and NaHS attenuates the development of hypertension and associated hypertensive heart disease. BP

reduction is the probable mechanism of action, but secondary effects such as reducing oxidative stress and fibrosis might also play a role. Furthermore, we are the first to show in a hypertensive rat model that the clinically applicable compound STS has promising cardioprotective properties.

For the administration of H₂S in experimental models, soluble sulfide salts are often used, while occasionally gaseous H₂S is administered. Recently, slow-release H₂S donors such as GYY4137 are becoming more popular. TS is an old player in the field, but recently rediscovered as a H₂S producing substance. TS is an intermediate of sulfur metabolism and a metabolite of H₂S that can also lead to the production of H₂S (Ubuka *et al.*, 2001; Olson *et al.*, 2013). In this study, we used STS because this is a non-toxic substance that is associated with the production of H₂S. A recent study showed that inhalation of H₂S markedly increased TS levels during endotoxaemia in mice. In addition, administration of STS dose-dependently improved survival after LPS challenge in mice (Tokuda *et al.*, 2012). The dosage of STS used in this study is based on previous experimental studies (Asplin *et al.*, 2009; Tokuda *et al.*, 2012; Shirozu *et al.*, 2014) and STS usage in humans (Cicone *et al.*, 2004; Farese *et al.*, 2011). The dosage range of STS in experimental studies is 0.5–2 g kg⁻¹ given daily, three times a week; in humans even 12.5 and 25 g of STS have been infused without adverse effects. In humans, the effect of i.v. STS infusion on plasma TS concentrations has been studied (Farese *et al.*, 2011). In our rat study, we did not measure plasma TS concentrations, to our knowledge there are no studies available that have done so in rats. It has to be taken into account that in our rat model, STS was given i.p. and not i.v., the kinetics of this route is not known. The clinical use of H₂S has not been attained, but STS has been used in clinical practice for decades. To date, short-term treatment with i.v. STS is safely used in patients for the treatment

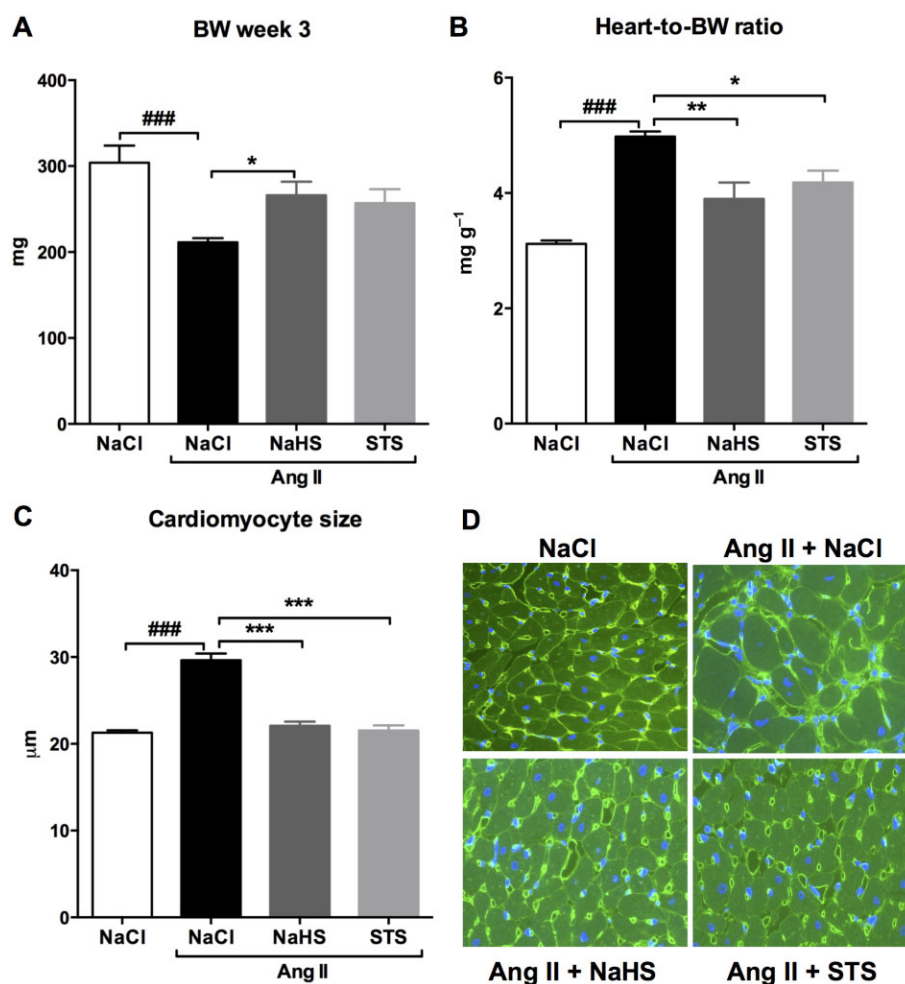


Figure 2

Treatment with STS and NaHS attenuated the cardiac hypertrophy induced by Ang II. (A) Ang II-infused vehicle-treated animals ($n = 7$) had a lower body weight than controls ($n = 6$). Simultaneous treatment with NaHS ($n = 6$), but not with STS ($n = 7$), resulted in a higher body weight compared with Ang II-infused vehicle-treated animals. (B) After 3 weeks of Ang II infusion the heart-to-body weight ratio was increased in vehicle-treated rats ($n = 7$) compared with 0.9% NaCl-infused controls ($n = 6$). Treatment with STS ($n = 7$) and NaHS ($n = 6$) attenuated the increase in heart weight. (C) To confirm this finding, we measured the average cardiomyocyte size in WGA-stained cardiac sections. STS ($n = 7$) as well as NaHS ($n = 6$) prevented the Ang II-induced increase in cardiomyocyte size. (D) Representative photomicrographs of WGA-stained cardiac sections. (### $P < 0.001$ vs. control, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs. Ang II + NaCl).

of calciphraxis (Singh *et al.*, 2011). However, one of the disadvantages for long-term administration might be the route of delivery, as orally administered STS is quickly degraded in the stomach and a validated oral form of the compound has not been developed yet. Furthermore, the long-term effects of STS administration should be further explored.

A substantial body of literature demonstrates the cardio-protective effects of H₂S. Many of these studies focused on the beneficial effects of H₂S in acute myocardial infarction induced by coronary artery ligation (Szabo *et al.*, 2011). There is only one study describing beneficial effects of STS in a hypotensive high-cardiac output model (Sen *et al.*, 2008). In humans, the effects of STS on BP have not been demonstrated extensively; however, there are indications that STS exhibits vasodilating effects (Thomas and McGinnis, 2002; Hayden *et al.*, 2005; Hayden and Goldsmith, 2010). We observed that

STS has similar protective effects as NaHS thereby providing us with exciting possibilities for the translation into clinical use.

The reduction in BP observed in this study is in line with previous findings showing the vasorelaxing properties of H₂S. Homozygous CSE-knockout and heterozygous CBS-deficient mice both develop hypertension (Yang *et al.*, 2008; Sen *et al.*, 2010), suggesting a role for endogenous H₂S production in the pathogenesis of hypertension. Furthermore, exogenous administration of H₂S causes vasodilatation in experimental models of hypertension (Zhong *et al.*, 2003; Mustafa *et al.*, 2011; Roy *et al.*, 2012). A proposed mechanism is the activation of K_{ATP} channels through sulfhydration (Mustafa *et al.*, 2011), but it is also suggested that the increased bioavailability of NO might play a role in the vasodilator effects of H₂S (Zhao and Wang, 2002). However, we observed that the anti-hypertensive effect of H₂S was not accompanied by an

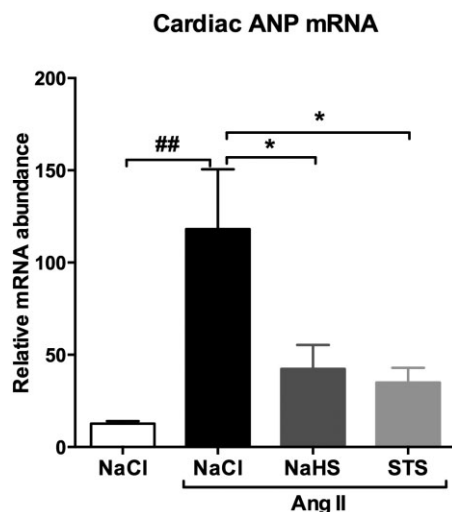


Figure 3

Reduced cardiac mRNA levels of ANP in STS- and NaHS-treated rats. Cardiac mRNA levels of ANP were ninefold increased after 3 weeks of Ang II infusion ($n = 7$) compared with 0.9% NaCl-infused controls ($n = 6$). Treatment with STS ($n = 7$) and NaHS ($n = 6$) reduced ANP mRNA expression by 70% and 64% respectively. (## $P < 0.01$ vs. control, * $P < 0.05$ vs. Ang II + NaCl). ANP mRNA levels were normalized to GAPDH.

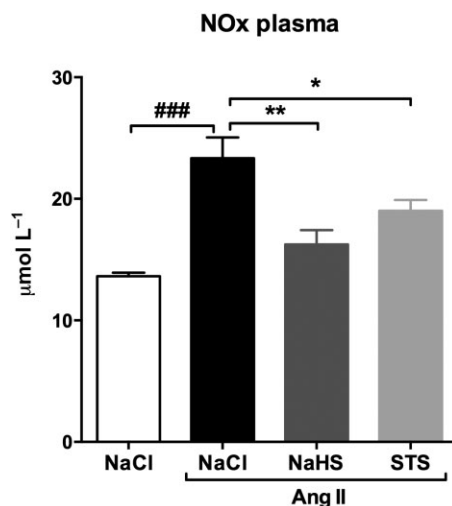


Figure 4

Ang II infusion induces a rise in plasma NOx levels. Plasma NOx levels were increased in Ang II-infused animals treated with vehicle ($n = 7$). In STS- ($n = 7$) and NaHS-treated ($n = 6$) animals, we observed no significant increase in plasma NOx levels. (### $P < 0.001$ vs. control, ** $P < 0.01$, * $P < 0.05$ vs. Ang II + NaCl).

increase in plasma NOx as compared with Ang II-infused animals suggesting that the vasodilator effects of H₂S are not related to an increased bioavailability of NO in our model. The increased plasma levels of NOx in the Ang II-infused animals treated with vehicle might be compensatory and due to the high BP. The ability of H₂S to reduce renin and the

activity of ACE also contributes to its BP reducing effects (Lu *et al.*, 2010). However, the contribution of this mechanism in this particular model is probably low or undetectable because of continuous Ang II infusion and its negative feedback effect on the production of renin.

In our model, the prevention of hypertension by STS and NaHS is probably the basis of the attenuation of cardiac hypertrophy. In the STS- and NaHS-treated groups, we observed a concordant smaller cardiomyocyte size and a lower heart-to-body weight ratio compared with untreated animals. The lower expression of ANP mRNA in these groups further supports the unloading of the heart. In overload-induced models of heart failure, treatment with H₂S attenuated the development of hypertrophy. Conversely, CSE-knockout mice with little endogenous H₂S production showed an increase in cardiac hypertrophy compared with wild-type mice (Kondo *et al.*, 2013).

Although we postulate that BP regulation is the primary mechanism of action in our model, it is known that Ang II causes damage independent of an elevated BP (Mervaala *et al.*, 2000). This enables additional protective modes of action of H₂S. We observed that treatment with H₂S influences cardiac fibrotic pathways as evidenced by a reduction in fibronectin mRNA and Masson-positive surface area. It is not certain if H₂S has any direct effects in fibrosis. Recent studies have demonstrated a potential link between the Ang II-associated fibrotic response and NADPH oxidases in cardiac fibroblasts (Lassegue and Griendling, 2010). Therefore, the antifibrotic effects of H₂S might be related to the reduction in Cybb (Nox2) we observed in this study. However, we also observed a reduction in galectin-3 mRNA, a protein with established fibrotic properties (de Boer *et al.*, 2009). Recently, it was shown that genetic and pharmacological inhibition of galectin-3 prevents cardiac remodelling by interfering with myocardial fibrogenesis (Yu *et al.*, 2013). Ang II-induced cardiac fibrosis was significantly decreased in galectin-3 knockout mice (Yu *et al.*, 2013).

Ang II induces oxidative stress by activating NADPH oxidases via the angiotensin-1 receptor (Agarwal *et al.*, 2004). H₂S is a known antioxidant and can directly scavenge ROS, increase intracellular glutathione levels and reduce the amount of ROS produced through modulation of mitochondrial ROS production (Kimura and Kimura, 2004; Kimura *et al.*, 2010; Bos *et al.*, 2013). In concordance with previous findings, we observed that treatment with NaHS reduced oxidative stress as evidenced by low Cybb (Nox2) mRNA and urinary MDA levels. These antioxidant effects may also have contributed to the protective effects of STS and NaHS in this study. The production of ROS because of the abundant presence of Ang II causes endothelial dysfunction and can therefore also contribute to the development of hypertension (Rathaus and Bernheim, 2002). Accordingly, treatment with antioxidants is able to reduce BP in experimental models of hypertension (Mullan *et al.*, 2002; Racasan *et al.*, 2004).

From our data, we are unable to deter whether the protective effects of STS can be completely attributed to the conversion of H₂S or if there are direct effects of STS. One of the proposed direct effects of STS is calcium chelation based on the fact that there are studies showing that calcium chelation inhibits some effects of Ang II (Brinson *et al.*, 1998; Du *et al.*, 1999). However, in contrast to what has been published

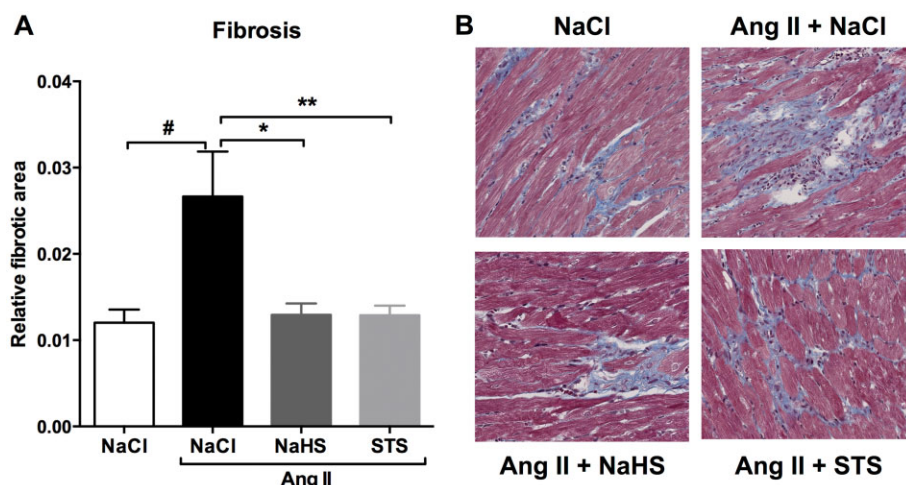


Figure 5

STS and NaHS treatment prevented the development of Ang II-induced cardiac fibrosis. (A) The relative fibrotic area was 2.2-fold increased in hearts of Ang II-infused animals treated with vehicle ($n = 7$) compared with 0.9% NaCl-infused control animals ($n = 6$). Treatment with STS ($n = 7$) and NaHS ($n = 6$) attenuated the deposition of collagens. (B) Representative photomicrographs of Masson stained cardiac sections. ($\#P < 0.05$ vs. control, $**P < 0.01$, $*P < 0.05$ vs. Ang II + NaCl).

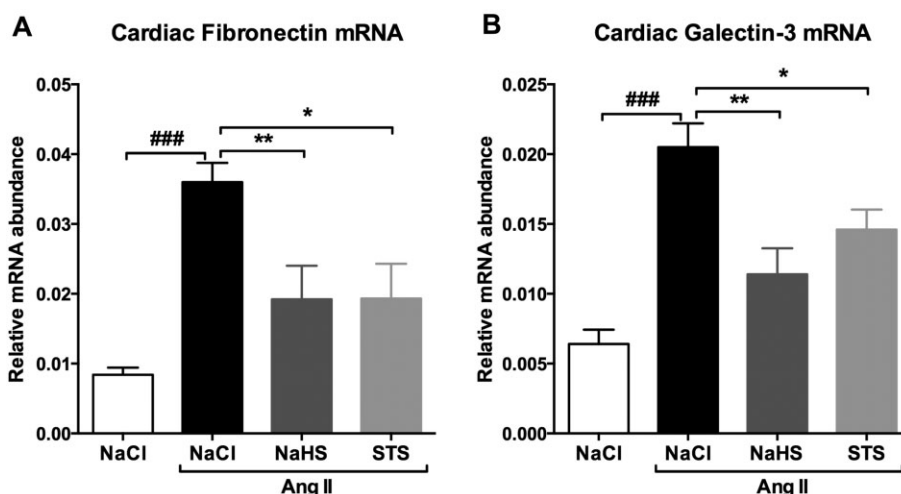


Figure 6

Treatment with STS and NaHS prevents the up-regulation of fibronectin and galectin-3. (A) Fibronectin and (B) galectin-3 mRNA levels were 4.3-fold and 3.2-fold increased in Ang II-infused animals treated with vehicle ($n = 7$) respectively. In STS- ($n = 7$) and NaHS-treated ($n = 6$) animals, no increase in fibronectin and galectin-3 mRNA levels were observed. ($###P < 0.001$ vs. control, $**P < 0.01$, $*P < 0.05$ vs. Ang II + NaCl). Fibronectin and galectin mRNA levels were normalized to GAPDH.

repeatedly, other studies show that STS is not a strong calcium chelator (Pasch *et al.*, 2008; O'Neill and Hardcastle, 2012). Our data indicate that calcium chelation did not play a major role in the protective effects of STS in this model. However, the calcium excretion measurements (Figure 8B) are possibly underpowered, so we must be careful in drawing firm conclusions from these data.

In this model, the beneficial effects are probably related to a reduction in BP, but also the attenuation of oxidative stress

and fibrosis may contribute to the protection against cardiac remodelling. We have extended these findings by demonstrating that STS has marked protective properties, which brings us closer to translation into clinical use. Whether the protective effects of STS are solely mediated by the conversion to H_2S or by additional direct effects of STS is currently not known. H_2S is a promising agent in the battle against hypertensive heart disease and might be a valuable addition to the already existing cardioprotective therapies.

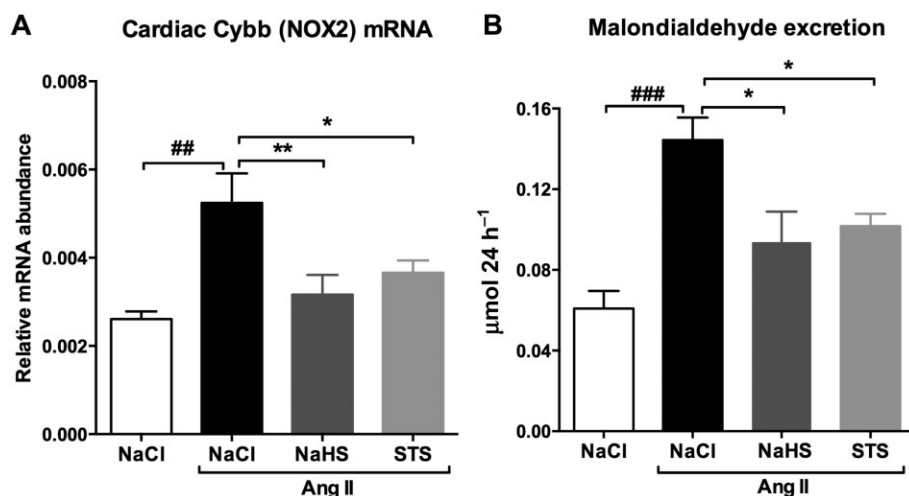


Figure 7

Treatment with NaHS and STS reduces oxidative stress. (A) Cardiac levels of Cybb (Nox2) mRNA and (B) urinary MDA levels were increased in vehicle-treated animals after 3 weeks of Ang II infusion ($n = 7$). Simultaneous treatment with either STS ($n = 7$) or NaHS ($n = 6$) prevented the development of oxidative stress, as evidenced by near-control Cybb (Nox2) mRNA and urinary MDA levels. (### $P < 0.001$, ## $P < 0.01$ vs. control, ** $P < 0.01$, * $P < 0.05$ vs. Ang II + NaCl). Nox2 mRNA levels were normalized to GAPDH.

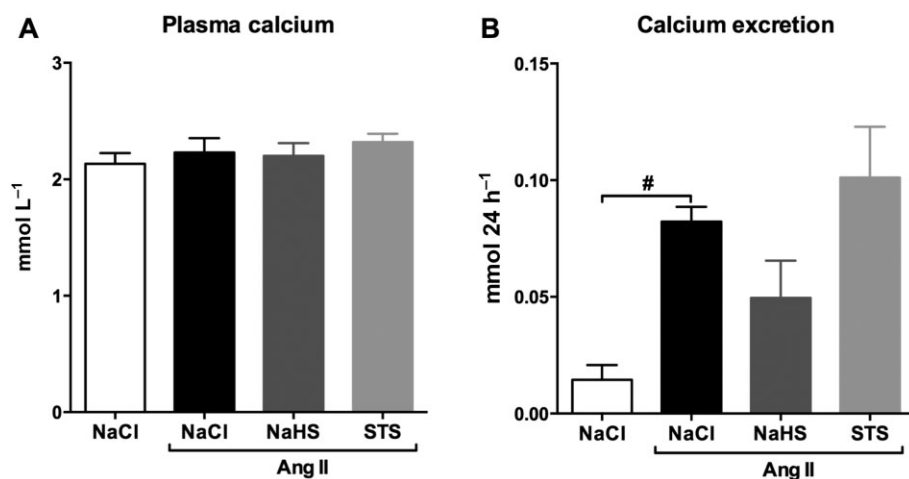


Figure 8

Unchanged urinary calcium levels upon STS treatment. (A) Plasma calcium levels were unaltered upon infusion with Ang II ($n = 7$) and simultaneous treatment with NaHS ($n = 6$) and STS ($n = 7$). (B) After 3 weeks of Ang II infusion ($n = 7$), the calcium excretion was increased compared with controls ($n = 6$). Urinary calcium levels from STS- ($n = 7$) and NaHS-treated ($n = 6$) animals were not different from Ang II-infused animals treated with vehicle. (# $P < 0.05$ vs. control).

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Author contributions

P. M. S., A. R. F., R. A. d. B., A. P., J. L. H., H. G. D. L. and H. v. G. designed the research; P. M. S., A. R. F., H. G. D. L. and H. v. G. performed the research; R. A. d. B., A. P. and J. L. H. contributed the new reagents/analytic tools; P. M. S., A. R. F., R. A. d. B., A. P., J. L. H., H. G. D. L. and H. v. G. analysed the data; P. M. S., A. R. F. and H. v. G. wrote the paper.

Conflicts of interest

A. P. has support from an unrestricted research grant provided from Köhler Chemie. The other authors declare no conflicts of interest.

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